

RESEARCH HIGHLIGHT

RNA modified with acyclic threoninol nucleic acids for RNA interference

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Although synthetic small interfering RNA (siRNA) has been extensively used to downregulate any protein-coding mRNA, several key issues still remain unsolved. The acyclic threoninol nucleic acid (aTNA), placed at certain siRNA positions, is a useful modification to reduce the oligonucleotides vulnerability towards nucleases. In addition, it can be exploited to avoid several OFF-target effects that limit the biological safety of the RNAi-based agents.

Keywords: RNA interference; siRNAs; acyclic nucleic acids

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In attempt to control gene expression, synthetic oligonucleotide derivatives have been found to be appealing agents with improved therapeutic potential^[1]. Antisense, siRNA and aptamers are well-established strategies to downregulate the expression of candidate genes. For example, siRNA-mediated silencing, taking advantages of the RNAi pathway and its protein complex (RISC), is a robust and potent strategy to trigger Post Transcriptional Gene Silencing (PTGS)^[2]. Hence, siRNA-based therapeutics have attracted the attention as promising strategy for clinical purposes^[3]. Recent therapeutic attempts have demonstrated the efficacy of the intravenous administration of siRNA molecules against cancer progression and metastasis^[4]. SiRNA molecules consist of two strands: the antisense and the sense, being the antisense or guide strand selected and loaded into the RISC complex. The complementarity between the antisense strand and a specific mRNA guides the efficient degradation of the specific mRNA, decreasing the amount of the corresponding protein^[5]. But despite the immense potential, siRNA technology has several disadvantages.

Unmodified oligonucleotides are promptly degraded by bloodstream and cellular endo-/exo-nucleases. In addition, the polyanionic character of both cell membranes and nucleic acids makes problematic the cellular uptake. Finally OFF-target effects such as saturation of RNAi machinery, misloading of the passenger strand and activation of the interferon response limit the biological safety of siRNA-based therapeutics. Most of these disadvantages may be solved by using modified nucleic acids^[6].

Few years ago we reported that a modified siRNA was clearly better than the corresponding unmodified siRNA in a mouse model of inflammatory bowel disease (IBD)^[7]. This modified siRNA was protected with a propanediol molecule at the 3' termini and two residues of 2'-methylated-RNA at the other end of the sense strand (5'). The large increase on efficacy and the relatively simplicity of the modifications triggered our interest in modified siRNA with enhanced stability toward nucleases. Thus, we introduced several hydrophobic groups in the 3'-termini of siRNAs. These

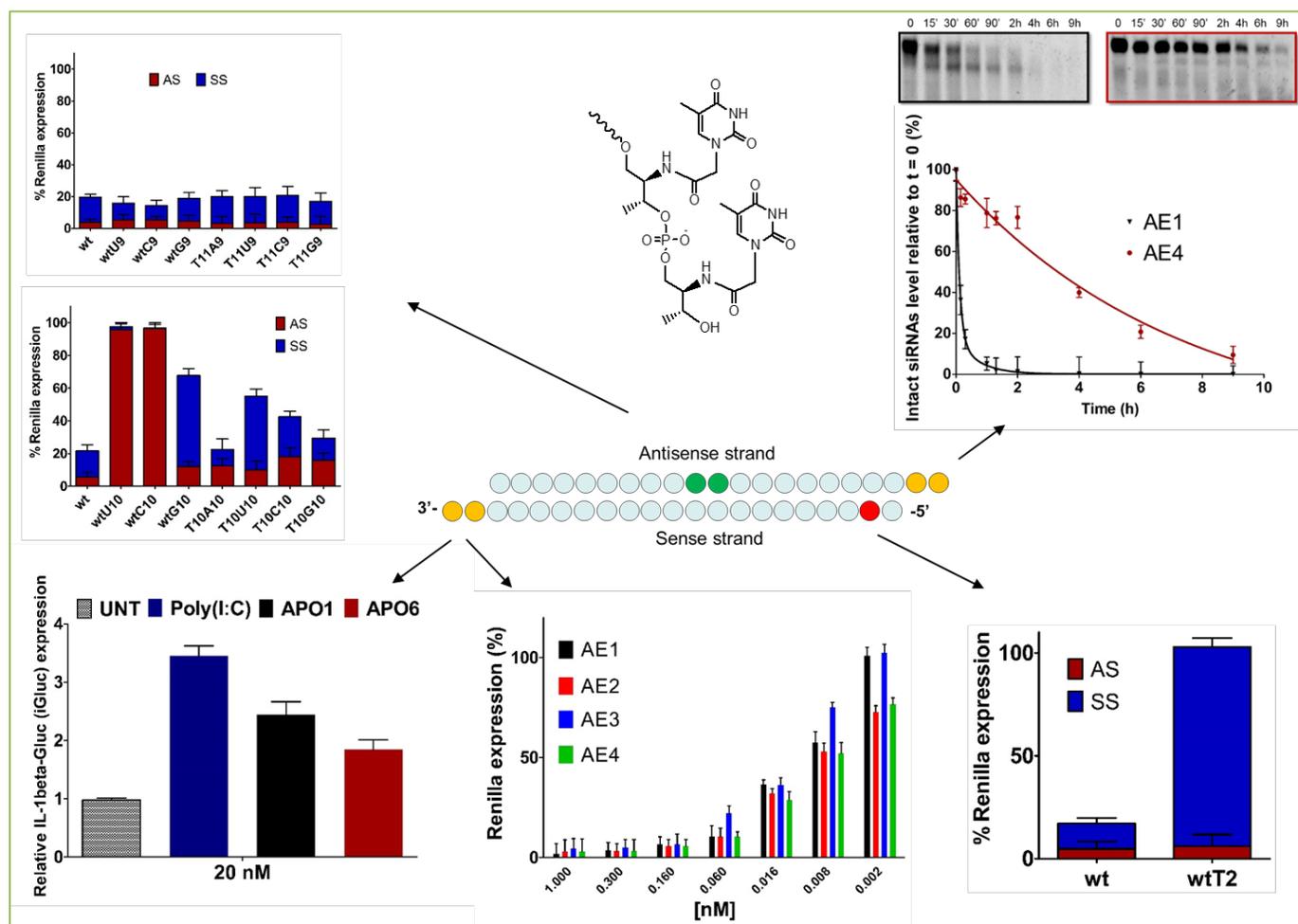


Figure 1. Beneficial effects of the substitution of natural nucleobases by L-threoninol derivatives in siRNAs. Adapted from references [15, 17].

modifications were designed to protect oligonucleotides toward nuclease degradation and fit into the hydrophobic pocket of RISC increasing the binding affinity of siRNA strands for RISC [8]. We observed that the smaller group in the antisense strand the more silencing of the corresponding siRNA. On the contrary the same modification in the 3'-termini of the sense strand did not provide any substantial variation of the silencing activity. These interesting conclusions inspire us to design other new modifications for siRNA derivatives.

For example, the *N-N* coupled nucleosides represent a new step in the development of novel nuclease resistant siRNAs [9]. The connection between two nucleosides occurs through the bases and not through the conventional phosphodiester linkage. Computational analysis of Klenow's active site complexed with the *N-N* coupled nucleosides has revealed that the extreme resistance towards nucleases depends essentially on the lack of the scissile phosphate [9].

Another structural parameter to take into account is the pucker of the ribose. In double-helical B-DNA, the pucker of the furanose is "South" (*S*). But double-helical DNA in A-conformation and RNA are characterized by the "North" (*N*) pucker of *N*-type conformation. Several authors concentrate their research efforts in the preparation of siRNA carrying nucleosides that stabilize the *North* conformation. Among them, the locked nucleic acids (LNA) [10], the *arabino* nucleic acids (ANA) [11] and the 2'-fluoroarabino (FANA) have disclosed interesting properties for further applications [12]. We have investigated the effects of the *N*-type carbocyclic pseudo-nucleoside derivatives on RNA interference experiments [13]. Some pseudo-nucleoside modifications at both siRNA strands have disclosed enhanced resistance to degradation; furthermore the inhibitory properties of siRNA duplexes remained substantially unchanged.

Recently, new paths have been beaten, instead of using "constrained" modifications able to preserve the A-form of RNA duplexes, more flexible acyclic derivatives have been

successfully used ^[14-16], to improve the stability towards nucleases and some biological properties. Specifically, we explored the effects of the acyclic L-threoninol backbone on the biological properties of siRNAs. The introduction of 2 units L-threoninol modification at the 3'-protuding ends of siRNAs confers stronger nuclease resistance, less activation of the interferon response and greater potency respect to unmodified siRNA molecules ^[15] (Figure 1 *upper right corner, lower left corner and central lower part, respectively*). The promising results encourage us to further analyse the L-threoninol backbone inside the siRNA duplex ^[17]. Because the L-threoninol-thymine showed high destabilization potential at internal position of duplex RNA ($\Delta T_m \approx 10$ °C respect to native one), we thought to investigate how central modifications could affect the potency, for example helping the unwinding or hindering the proper activity of the slicer Ago2. We placed L-threoninol on positions crucial for the cleavage of the target mRNA: 10 and 11 of the antisense strand. In case of modification on position 10 the potency was strongly reduced (≈ 10 -fold), on the other hand the modification of 11th position led to a slight decrease of potency respect to unmodified siRNAs (Figure 1 *upper left corner*). Hence, depending on position, L-threoninol modification interferes with correct and catalytically competent conformation of the Ago2 active site.

Besides improved biological properties such as the resistance towards nucleases and sustained potency; we concentrate our efforts in the reduction of the OFF-target effects. One important source of these OFF-target effects comes from the wrong selection of the strand by RISC. Ratio between antisense and sense target activity can be assessed employing PsiCHECK2 reporter. Measuring the targeting ability of each strand independently, it provided rapid and reliable estimation on the efficiencies of siRNA-mediated inhibition for both the antisense (AS) and sense strand (SS). First, we examined whether L-threoninol modification at central positions, may induce a strong bias toward the correct guide-mediated silencing. Again, depending on position and also the mismatches type facing the L-threoninol unit, the ratio between AS and SS silencing is poor or increased. Finally, we thought to introduce of L-threoninol modification at the 2nd position of the 5'-termini of the passenger strand (Figure 1 *lower right corner*). The position 2 is important for the initial recognition of the loaded strand with the complementary mRNA. Disrupting or hampering the initial base pairing interactions could induce an inefficient assembly of the RISC complex and so the flawed processing of the mRNA complementary to the sense strand. The presence of single L-threoninol entailed complete abrogation of the passenger-mediated silencing without affecting the guide-mediated silencing.

In conclusion, minimal, cost-effective L-threoninol modification, disclosing strong impact on potency, nucleases resistance, interferon response and unwanted passenger-mediated silencing, paves the way to valuable strategy in the tailored design of therapeutic siRNAs.

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